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Persistence of Enterohemorrhagic *Escherichia coli* O157:H7 in Soil and on Leaf Lettuce and Parsley Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water

MAHBUB ISLAM,[1] MICHAEL P. DOYLE,[1]* SHARAD C. PHATAK,[2] PATRICIA MILLNER,[3] AND XIUPING JIANG[4]

[1]*Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223-1797;* [2]*Department of Horticulture, University of Georgia, Coastal Plain Experiment Station, Tifton, Georgia 31793;* [3]*Animal Waste Pathogens Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Building 001, Beltsville, Maryland 20705-2350; and* [4]*Department of Food Science and Human Nutrition, Clemson University, Clemson, South Carolina 29634, USA*

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ABSTRACT

Outbreaks of enterohemorrhagic *Escherichia coli* O157:H7 infections associated with lettuce and other leaf crops have occurred with increasing frequency in recent years. Contaminated manure and polluted irrigation water are probable vehicles for the pathogen in many outbreaks. In this study, the occurrence and persistence of *E. coli* O157:H7 in soil fertilized with contaminated poultry or bovine manure composts or treated with contaminated irrigation water and on lettuce and parsley grown on these soils under natural environmental conditions was determined. Twenty-five plots, each 1.8 by 4.6 m, were used for each crop, with five treatments (one without compost, three with each of the three composts, and one without compost but treated with contaminated water) and five replication plots for each treatment. Three different types of compost, PM-5 (poultry manure compost), 338 (dairy manure compost), and NVIRO-4 (alkaline-stabilized dairy manure compost), and irrigation water were inoculated with an avirulent strain of *E. coli* O157:H7. Pathogen concentrations were 10^7 CFU/g of compost and 10^5 CFU/ml of water. Contaminated compost was applied to soil in the field as a strip at 4.5 metric tons per hectare on the day before lettuce and parsley seedlings were transplanted in late October 2002. Contaminated irrigation water was applied only once on the plants as a treatment in five plots for each crop at the rate of 2 liters per plot 3 weeks after the seedlings were transplanted. *E. coli* O157:H7 persisted for 154 to 217 days in soils amended with contaminated composts and was detected on lettuce and parsley for up to 77 and 177 days, respectively, after seedlings were planted. Very little difference was observed in *E. coli* O157:H7 persistence based on compost type alone. *E. coli* O157:H7 persisted longer (by >60 days) in soil covered with parsley plants than in soil from lettuce plots, which were bare after lettuce was harvested. In all cases, *E. coli* O157:H7 in soil, regardless of source or crop type, persisted for >5 months after application of contaminated compost or irrigation water.

Escherichia coli is a normal member of the gastrointestinal microflora of humans and animals; however, enterohemorrhagic *E. coli* strain O157:H7 can cause severe hemorrhagic disease and death in humans *(4, 29)*. Since the first outbreak of *E. coli* O157:H7 infection in 1982, there have been many outbreaks in the United States, with estimates of approximately 73,000 *E. coli* O157:H7 infections and 61 associated deaths occurring annually *(7)*. Although undercooked ground beef has been identified as the leading food vehicle of *E. coli* O157:H7 infections, fresh fruits and vegetables and raw unpasteurized juice are becoming increasingly important vehicles of foodborne transmission *(2, 27)*.

Many outbreaks of *E. coli* O157:H7 infections associated with contaminated lettuce and a large outbreak associated with the consumption of contaminated unpasteurized apple juice have been reported *(7)*. The potential for widespread outbreaks of human infection caused by consumption of contaminated raw produce was represented by an epidemic of *E. coli* O157:H7 infection associated with radish sprout consumption in Japan involving more than 9,000 cases and 12 deaths *(16)*. In July 1998, an outbreak of *E. coli* O157:H7 infection involving 40 Montana residents was associated with contaminated leaf lettuce *(1)*. Following this outbreak, at least four additional outbreaks of *E. coli* O157:H7 infection have implicated lettuce *(5)*. These outbreaks highlight the increasing importance of fresh produce as a vehicle of foodborne illness.

Vegetable plants can become contaminated with pathogens before harvest when grown in fields fertilized with fresh or inadequately composted manure *(33)*. Both conventional and organic vegetable producers commonly apply animal manure as fertilizer to fields where crops are grown *(28, 34)*. Healthy cattle sporadically harbor *E. coli* O157:H7 in their gastrointestinal tract and shed the pathogen asymptomatically in their feces *(20, 36)*. In the northern United States, the prevalence of *E. coli* O157:H7 carriage by cattle ranges from 6 to 9% *(11, 32, 37)*. *E. coli* O157:H7 may be present in up to 8.3% of dairy cattle *(11)*. Recent surveillance data indicate that prevalence rates of *E. coli* O157:H7 in cattle are much higher than those estimated several years ago. Elder et al. *(10)* found that of 29 lots of

* Author for correspondence. Tel: 770-228-7284; Fax: 770-229-3216; E-mail: mdoyle@uga.edu.

cattle tested, 72% had at least one fecal specimen that was positive for enterohemorrhagic *E. coli* O157 (H7 or nonmotile) and 38% had positive hide samples. The cattle were tested in late summer and early fall, corresponding to peak seasonal *E. coli* O157:H7 prevalence. One reason for this higher prevalence rate could be the use of more sensitive methods to detect the pathogen in environments with competitive flora. This high rate of *E. coli* O157:H7 carriage by cattle indicates the need for intervention strategies at the point of production to prevent contamination of food and water supplies. Previous studies have revealed long-term survival (up to 21 months) of *E. coli* O157:H7 in manure held under a variety of conditions *(21)*. In addition, *E. coli* O157:H7 can survive, replicate, and move within soil, and the presence of manure increases the pathogen's survival time *(13, 14)*.

E. coli O157:H7 may also be introduced into soil through irrigation water contaminated with cattle feces or through contact with contaminated surface runoff from cattle production operations *(3, 5)*. Several outbreaks of *E. coli* O157:H7 infection have been associated with contaminated water *(6)*. The organism can survive for many weeks in deionized water and lake water, depending upon temperature *(8, 35)*. Contaminated water used to irrigate lettuce fields was believed to be the source of a multistate outbreak of *E. coli* O157:H7 infection associated with the consumption of mesclun lettuce in 1996 *(17)*.

The increased association of fresh vegetables with outbreaks of *E. coli* O157:H7 infection has heightened concern regarding pathogen contamination of vegetables in the agricultural environment via animal manure or irrigation water. However, there is little information regarding *E. coli* O157:H7 survival in manure-amended soils and on vegetables grown on those soils under field conditions. The objective of this study was to determine the fate of *E. coli* O157:H7 on lettuce and parsley and in surrounding soil when different types of contaminated manure composts or irrigation water were applied to soil in field plots typical of those used for vegetable production.

MATERIALS AND METHODS

Bacteria. All composts and irrigation water were inoculated with nontoxigenic *E. coli* O157:H7 strain B6914 (J. S. Karns and J. V. Gagliardi, U.S. Department of Agriculture, Agricultural Research Service [USDA-ARS], Beltsville, Md.) *(12)*. This strain does not possess Shiga toxin I or II genes but does carry a stable plasmid with genes for green fluorescent protein and ampicillin resistance *(15)*, thereby enabling the differentiation and selection of *E. coli* O157:H7 colonies isolated from a complex soil substrate. These characteristics make this strain a suitable surrogate for Shiga toxin–producing *E. coli* O157:H7 in survival studies.

Inoculum preparation. *E. coli* O157:H7 strain B6914 cells were thawed from frozen stock culture, streaked onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, Md.) containing 100 μg/ml ampicillin, and incubated for 24 h at 37°C. A single colony from the TSA-ampicillin (TSA-A) plate was inoculated into 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson) containing 100 μg/ml ampicillin (TSB-A) and incubated at 37°C for 16 to 18 h with agitation (150 rpm) for growth to the mid-log phase. A 0.5-ml suspension of the isolate was transferred to 100 ml of TSB-A and incubated at 37°C for 16 to 18 h with agitation (150 rpm). The bacteria were three times, sedimented by centrifugation at 5,000 × *g* for 20 min, and washed with 0.1% peptone water. Cells then were resuspended in 0.1% peptone water to achieve an optical density of 0.5 at 630 nm (ca. 10^8 CFU/ml). Cells were enumerated on TSA-A plates incubated at 37°C for 24 h.

Compost preparation. Composts PM-5 (poultry manure compost), 338 (dairy manure compost), and NVIRO-4 (alkaline-stabilized dairy manure compost) were obtained from the USDA (Agricultural Research Center, Beltsville, Md.). Dairy manure and poultry litters were used to prepare the composts. The manures were mixed with organic bulking materials or inorganic by-products in different proportions. Water was added initially as needed to bring the moisture content to within 40 to 50%. The dairy cattle manure from the 200-head Holstein herd at the USDA-ARS facility was collected from a sawdust-bedded, free-stall barn. Solids were separated from the liquid for composting. Dairy manure compost 338 contained dairy solids, straw bedding material, and old silage from the dairy barn. Alkaline-stabilized NVIRO-4 compost contained dairy manure solids, wood chips, and leaves at a ratio of 10:5:1 and added 3.6% Fe^{2+} and 25% fly ash. PM-5 compost was prepared from poultry manure (without bedding but including feathers) from a commercial egg-laying operation in Pennsylvania. All composting was done at the Beltsville Composting Research Facility. PM-5 contained leaves, poultry manure, crushed Christiana clay, and straw at a ratio of 12:10:6:3. Piles were composted in heaps for a total of 5 months *(30)*.

Vegetable production conditions. Production practices outlined in the *Handbook for Vegetable Growers (24)* were followed for growing leaf lettuce (*Lactuca sativa* L.) and parsley (*Petroselinum crispum* Mill.) at the Horticulture Farm fields of the Coastal Plain Experiment Station (University of Georgia, Tifton). Commercial seedling producers (Gardensmith Greenhouse, Jackson, Ga.; Taylor Farms, Tifton, Ga.) supplied the 2-week-old seedlings of parsley (variety Esmeralda) and leaf lettuce (variety Cardinale), respectively. Each of the three poultry and dairy manure composts was inoculated with *E. coli* O157:H7 at 10^7 CFU/g. Composts were applied as a strip at the rate of 4.5 metric tons per hectare in late October on the day before transplantation of seedlings. Contaminated irrigation water was inoculated with 10^5 *E. coli* O157:H7 cells per ml and was sprayed with a hand sprayer on each of the five plots for each crop only once as a treatment at the rate of 2 liters per plot 3 weeks after the seedlings were transplanted. No chemicals were used for weed control because of the possible lethal effect on the microorganisms. A split-plot block design plan was followed for each crop, with five treatments (one without compost, three with each of three composts, and one without compost but with contaminated water applied) and five replicates for a total of 25 plots for each crop. Each plot measured 1.8 by 4.6 m.

Soil and vegetable sample collection. At appropriate time intervals, ca. 100 g of soil surrounding a randomly selected plant at a depth of 2.5 cm from the surface was aseptically collected in a sterile plastic bag from each plot for each crop. Collection of plant samples began on day 21, when the plants were large enough for sampling. From each plot, approximately 5 g of lettuce or parsley leaves was obtained aseptically from a randomly selected plant and placed in a sterile plastic bag. The samples were brought to the laboratory from the field in a cooler with ice packs, held at 4°C, and analyzed within 48 h.

Enumeration of *E. coli* O157:H7. TSA-A was used for selective isolation of *E. coli* O157:H7 B6914 from soil and vege-

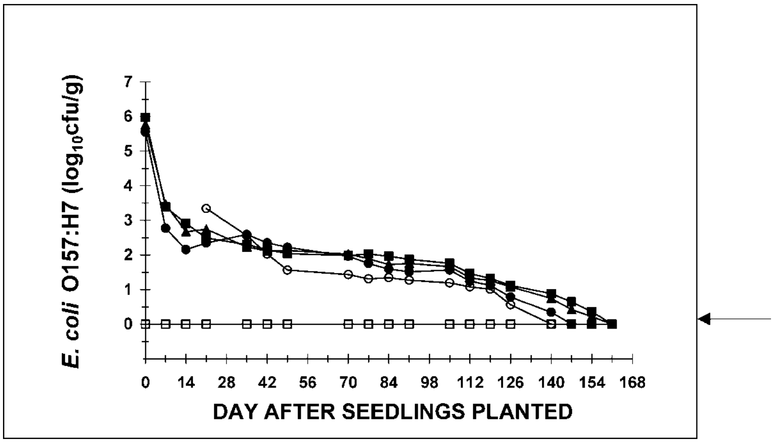


FIGURE 1. *Survival of* E. coli *O157:H7 in soil samples from lettuce fields amended with inoculated compost or irrigated with inoculated water. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Contaminated irrigation water was applied 3 weeks after seedlings were planted. Arrow (←) indicates not detectable by enrichment culture.*

table samples. Each soil sample (10 g) in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, Wis.) with 90 ml of 0.1% peptone water was pummeled in a Stomacher 400 laboratory blender (Teckman, Cincinnati, Ohio) for 30 s at low speed. Approximately 5 g of each lettuce or parsley sample was added to 45 ml of 0.1% peptone water in a sterile Whirl-Pak bag and rinsed by rubbing and vigorously agitating by hand for 30 s. *E. coli* O157:H7 counts for soil and vegetable samples were determined as follows. Serial dilutions (1:10) of each sample were prepared with 0.1% peptone water, and 0.1-ml portions of each dilution were spread onto TSA-A plates. Plates were incubated at 37°C for 24 h, and colonies of *E. coli* O157:H7 that fluoresced under UV light at 302 nm were counted. When the pathogen was not detected by direct plating, 1 g of soil sample or 1 ml of vegetable rinse suspension in 99 ml of universal preenrichment broth (Difco, Becton Dickinson) was incubated at 37°C for 24 h with agitation (150 rpm) for enrichment culture. Dilutions (1:10) of enrichment cultures were surface plated on to TSA-A plates and observed for colonies of *E. coli* O157:H7 following incubation.

Analyses of pH and moisture. The pH of manure-amended soil was determined by adding 10 g of soil to 250 ml of distilled water. The suspension was stirred for 5 min and then allowed to settle for 5 min. The pH of the liquid was determined with an Accumet Basic pH meter (Fisher Scientific, Pittsburgh, Pa.). Moisture content of manure-amended soil was determined by drying 5 g of soil at 105°C for 24 h in a drying oven (Precision Scientific, Winchester, Va.) and then weighing the residual.

Statistical analyses. The experimental design was a split-plot of which crop was the main plot and treatment was the subplot. Each treatment was replicated five times, and each sample from a treatment was plated in duplicate at each sampling time. Hence, *E. coli* O157:H7 results reported for each data point represent the mean of 10 values. *E. coli* O157:H7 not detected by initial plating and enrichment culture were treated statistically as zero. Data were analyzed by the general linear model procedure of the Statistical Analysis System *(31)*.

RESULTS

E. coli O157:H7 persisted for 154 and 217 days in the amended soil samples on which both lettuce (Fig. 1) and parsley (Fig. 2) were grown, respectively. Types of contaminated composts did not affect the persistence of *E. coli* O157:H7 in these soils. *E. coli* O157:H7 was detected for 77 and 177 days on lettuce (Fig. 3) and parsley (Fig. 4), respectively, following transplantation of seedlings. Mineral and nitrate compositions of the three different types of composts (PM-5, 338, and NVIRO-4) were determined in a commercial soil testing laboratory (Table 1). Nitrogen-phosphate-potassium values of the composts were highest for 338 and lowest for NVIRO-4 compost. The pH values for PM-5, 338, and NVIRO-4 composts were 8.1, 8.7, and 7.5, respectively. Throughout the study period of 266 days, pH of the manure compost-amended soil (data not shown) for both lettuce and parsley under all the treatments ranged between 5.7 and 8.3. Moisture contents of the compost-amended soil (data not shown) varied widely from <1 to 12.2%, depending on the rainfall. Harvest data (not shown)

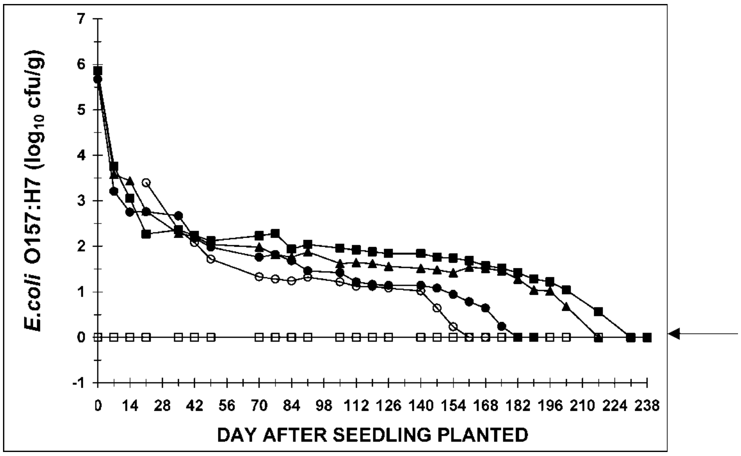
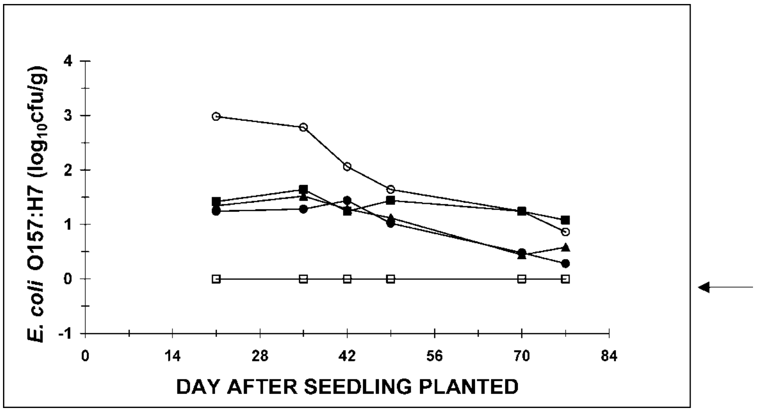


FIGURE 2. *Survival of* E. coli *O157:H7 in soil samples from parsley fields amended with inoculated compost or irrigated with inoculated water. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Contaminated irrigation water was applied 3 weeks after seedlings were planted. Arrow (←) indicates not detectable by enrichment culture.*

FIGURE 3. E. coli *O157:H7 counts on lettuce grown in fields amended with inoculated compost or irrigated with inoculated water. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Contaminated irrigation water was applied 3 weeks after seedlings were planted. Arrow (←) indicates not detectable by enrichment culture.*



for the crops revealed that soil fertilized with alkaline-stabilized dairy manure compost provided the lowest yields of both lettuce and parsley, with the highest yields obtained from 338 compost-amended soil. At day 177 when parsley was harvested, the average weight of a parsley plant was 263, 300, and 363 g when grown in soil amended with NVIRO-4, 338, and PM-5, respectively. For lettuce, when harvested at day 77, the average weight of a leaf was 124, 134, and 199 g when grown in soil amended with NVIRO-4, 338, and PM-5, respectively.

DISCUSSION

An important finding of this work is that *E. coli* O157:H7 can survive for >7 months in soil of some vegetable fields exposed to southern fall-winter conditions. This finding provides a better understanding of the ecology of *E. coli* O157:H7 in the production of parsley and leaf lettuce. Our results indicate that appropriate management of farm waste is critical in controlling the spread of this pathogen to vegetable crops.

Outbreaks of *E. coli* O157:H7 infections historically have been associated with consumption of undercooked ground beef; however, many recent outbreaks have resulted from consumption of contaminated raw vegetables, including lettuce *(1–3, 9, 17).* Although many pathogens have been associated with fresh produce, *E. coli* O157:H7 is of particular concern because ingestion of relatively few cells can cause illness *(4, 29).* This pathogen is hardy and can survive for extended periods of time in water and soil, at freezing and refrigeration temperatures, and under acidic and dry conditions *(7).*

Microbial contamination can occur during any of the steps involved in vegetable production, harvesting, and packing. However, the steps in the production chain that have the greatest potential for pathogen contamination are soil preparation (use of uncomposted organic manures) and planting and growing (use of contaminated irrigation water and animal manures and manure from animals grazing locally or nearby) *(5).* Root crops and leafy vegetables have the greatest risk of contamination through manure applied to soil *(28).*

Drinking and recreational water have been vehicles of *E. coli* O157:H7 infection in several outbreaks, supposedly associated with fecal contamination by infected animals or humans *(6).* For example, a waterborne outbreak at the Washington County Fair in New York State in August 1999 resulted in 921 cases of *E. coli* O157:H7 infection, including 11 cases of hemolytic uremic syndrome and two deaths *(6).* A shallow well supplying unchlorinated water to several vendors of beverages and ice was likely contaminated by manure. We have determined in our study that lettuce and parsley grown in soil treated with irrigation water contaminated with *E. coli* O157:H7 results in contamination of the outer portion of these vegetable plants.

Our studies indicate that if *E. coli* O157:H7 reaches the soil surface through manure application, irrigation wa-

FIGURE 4. E. coli *O157:H7 counts on parsley grown in fields amended with inoculated compost or irrigated with inoculated water. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Contaminated irrigation water was applied 3 weeks after seedlings were planted. Arrow (←) indicates not detectable by enrichment culture.*

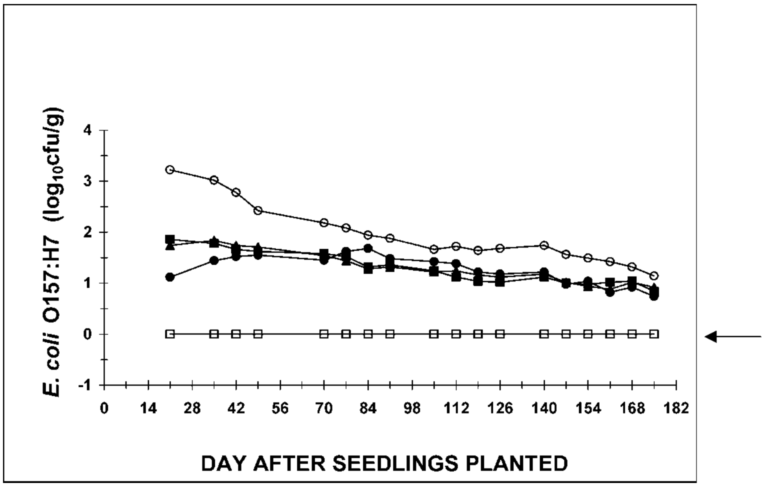


TABLE 1. *Mineral and nitrate composition and pH of compost preparation before application to soil*[a]

Compost[b]	Chemical composition (kg/ha of compost)									pH
	Cu	Fe	Mn	Zn	Ca	Mg	K	P	NO_3	
PM-5	1.3	3.5	13.3	1.9	5,400	411	16,600	333	1,700	8.1
338	12.2	58.3	73	31.5	9,200	813	55,700	357	7,000	8.7
NVIRO-4	1.2	3.6	57.8	4.8	8,100	402	3,330	62	185	7.5

[a] Average of four samples.

[b] PM-5 is a poultry manure compost, 338 is a dairy manure compost, and NVIRO-4 is an alkaline-stabilized dairy manure compost.

ter, or runoff from cattle operations, the pathogen has the potential to survive and move vertically with time. Rainfall may spread the pathogen through soil by runoff containing stored or unincorporated manure *(14)*. Soil characteristics also may play a role in the survival of *E. coli* O157:H7. The presence of manure can enhance the survival of *E. coli* O157:H7 in no-till soils *(13)* and in soil cores containing rooted grass, where after 130 days *E. coli* O157:H7 cell numbers only declined from 10^8 to 10^6 CFU/g of soil *(26)*. *E. coli* O157:H7 survived for 21 months under fluctuating temperature conditions in a manure pile collected from experimentally inoculated sheep *(21)*.

Survival of *E. coli* O157:H7 in the environment may play an important role in the persistence and dissemination of the pathogen on farms. Studies have revealed that *E. coli* O157:H7 inoculated into cattle feces remained detectable at high levels for >50 days, whereas in cattle slurry the pathogen declined by $>10^6$ CFU/ml in 10 days *(18, 22, 26)*. In studies of *E. coli* O157:H7 in microcosms simulating cattle water troughs, the pathogen survived for at least 245 days in the microcosm sediments *(23)*. Furthermore, *E. coli* O157:H7 strains surviving >6 months in such contaminated microcosms were able to persist in 10-week-old calves for 87 days postexposure. Water trough sediments contaminated with feces from cattle excreting *E. coli* O157:H7 may serve as long-term reservoirs of the pathogen on farms and as a source of infection for cattle *(23)*.

Many studies have addressed differences in *E. coli* O157:H7 survival rates in soils under a variety of conditions. Under laboratory conditions at 18°C, *E. coli* O157:H7 survived better in cores from a grass lawn, in which cell numbers decreased from 8.1×10^7 to 8.7×10^6 CFU/g after 63 days, than in cattle feces or slurry *(25)*. *E. coli* O157:H7 was less persistent in sieved grass-free soils than in intact soil cores containing rooted grass. Hence, vegetation appears to provide a protective environment for *E. coli* O157:H7 and enhances survival of this pathogen. In our study, *E. coli* O157:H7 also survived considerably longer in soil covered with parsley than in soil exposed to the environment after lettuce had been harvested.

E. coli O157:H7 has been isolated with increasing frequency from fresh produce, including leaf lettuce, but the modes by which the pathogen contaminates the plants remain unclear. Lettuce that has been fertilized with manure or irrigated with water contaminated with *E. coli* O157:H7 can take the bacteria up through the root system and internalize it in the leaves, thereby resisting traditional external sanitizing treatments *(33)*. Studies of planted lettuce in a mixture of soil and fresh cow manure inoculated with *E. coli* O157:H7 revealed that at maturation the pathogen had migrated to internal locations in plant tissue through the root system and could be seen throughout the edible portion of the lettuce. A similar transmission of the bacterium from contaminated irrigation water to lettuce leaves also was demonstrated *(33)*. In our study, results suggest that edible portions of the plant can become contaminated without constant direct exposure to *E. coli* O157:H7, perhaps through internal transport of the bacteria by the root system, by external physical means such as the wind or rain, or by initial contact with contaminated irrigation water and subsequent long-term persistence of the bacteria on the plants.

These studies have identified important factors that can contribute to *E. coli* O157:H7 contamination of produce and should be addressed by produce growers when managing their fields. Lettuce and parsley grown in soil containing contaminated manure or irrigated with contaminated water can become contaminated and remain so for several months. Our results indicate that contaminated irrigation water can play an important role in contaminating vegetables and the soil in which they grow. The origin and distribution of irrigation water and the history of the soil should be known to limit the introduction of pathogens to produce through irrigation water. Irrigation wells should be maintained properly to prevent pathogen contamination, and irrigation water sources should be monitored for microbiological safety. Long-term survival of *E. coli* O157:H7 in soil amended with manure compost illustrates the need for appropriate farm waste management to curtail environmental spread of this bacterium. Manure used as fertilizer or as a soil amendment should be treated to eliminate pathogenic microorganisms (e.g., composting or aging), and suitable buffer zones should be positioned between animal grazing areas and vegetable production fields. Based on this and previous *(19)* studies, an aging time of ≥8 months should be scheduled between the final manure application to vegetable growing fields and harvest.

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